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(54) Title: ACTIVE IMMUNIZATION AGAINST ANGIOGENESIS–ASSOCIATED ANTIGENS

(57) Abstract

Modified angiogenic self antigens and anti–idiotypic antibodies that mimic an antigenic determinant of a receptor to an angiogenic molecule are provided. *In vitro* and *in vivo* methods of using these antigens and antibodies are also provided.

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ACITVE IMMUNIZATION AGAINST ANGIOGENESIS-ASSOCIATED ANTIGENS

BACKGROUND OF THE INVENTION

Current immunotherapeutic approaches to inhibiting angiogenesis rely on the passive administration of large amounts of antibodies to angiogenesis targets such as the vitronectin receptor and vascular endothelial cell growth factor (VEGF). Limitations of this therapeutic strategy include the difficulty of administering large amounts of monoclonal antibodies to a patient and maintaining a constant high level of antibodies over a long period of time in a patient.

The present invention overcomes these problems by using active specific immunotherapy against angiogenesis target molecules to inhibit angiogenesis. Such methods of immunotherapy against angiogenic molecules include modification of immunogens to cause an immune response against the angiogenic molecules. Modification of the target antigen can be achieved by, for example, conjugation of immunogenic reagents to the antigen (see US Patent Nos. 5,334,379); haptenization of the antigen (see U.S. Patent Nos. 4,778,752 and 5,290,551); the use of adjuvants bound to, or administered with, the target antigen; binding peptide fragments to the antigen; binding the target antigen to MHC class I and class II restricted antigens (see U.S. Patent No. 4,478,823); changing glycosylation patterns of the target antigens (see U.S. Patent No. 5,484,735); presenting the target antigen on antigen presenting cells, such as dendritic cells (see U.S. Patent Nos. 5,580,563 and 5,788,963), among other methods.

Another method where immunity is induced to an angiogenesis target molecule

is that of using an anti-idiotypic antibody bearing the internal image of the target antigen. Since the mimicry of the target "self" antigen by the anti-idiotype is likely to be imperfect, such anti-idiotypes will break tolerance to a self antigen, although administration of the self antigen is not able to do so. It has been previously shown that anti-idiotypes that bear the internal image of carcinoembryonic antigen (CEA) can induce anti-CEA antibodies in patients with colorectal carcinoma, while CEA itself cannot do so. Therefore, an alternative approach to using the actual "self" angiogenesis target antigen as a vaccine is to use an anti-idiotypic antibody that mimics the antigen, bears an internal image of the antigen, and elicits an immune response.

An anti-idiotypic antibody, termed either an AB2 or an anti-idiotype, is one produced in response to the presence of an antibody in an immunologically active system. Anti-idiotypes are antibodies directed against the antigen-combining region or variable region (known as the idiotype) of another antibody molecule.

The theory of idiotypic relationships and networks is based on the Jerne model (Jerne, N.K. (1974) Ann. Immunol. (Paris) 125C: 373; Jerne, N.K. et al. (1982) EMBO 1:234). Thus immunization with an antibody expressing a paratope (antigen combining site) for a given antigen, should result in anti-antibodies (anti-idiotypes), some of which share with the antigen a complementary structure to the paratope. Those anti-idiotypes could then possibly act as antigens, i.e., mimic the antigen. Thus, the immune system would carry within it an internal image of the antigen, the anti-idiotype.

Anti-idiotypic antibodies have been studied as potential vaccines against pathogenic organisms (Kennedy, R. C. et al., 1986, Science 232:220; Reagan, K. J. et al., 1983, J. Virol. 48:660; McNamara, M. K. et al., 1984, Science 226:1325; Sachs, D. L. et al., 1982, J. Exp. Med. 155:1108) and malignant tumors (Chen, Z. J. et al., 1991, J. Immunol. 147(3):1082; Dunn, P. L. et al., 1987, J. Immunol. 60:181-187; Herlyn, D. et al., 1987, Proc. Natl. Acad. Sci. USA 84:8055; Chattopadhyay, P. et al., 1991, Cancer Res.

51:3183). In animal studies, murine anti-idiotypic antibodies have demonstrated antigen-specific responses across species (xenogeneic model) (Chapman, P. B. and A. N. Houghton, 1991, J. Clin. Invest. 88:186) and within the same inbred species (syngeneic model) (Gaulton, G. N. et al., 1986, J. Immunol. 137:2930; Chen, Z. J. et al., 1991, J. Immunol. 147(3):1082;).

Although anti-idiotypic antibodies of angiogenic factors have been developed (WO/9608513, published March 21, 1996), including those for modulation of tumor progression (Ortega, N. et al., C. R. Acad. Sci. III (FRANCE), May 1996, 319(5):411-415), there has been no disclosure prior to this invention of the use of anti-idiotypic antibodies mimicking angiogenic factor receptors, i.e., integrins, and VEGF receptors such as basic FGF receptor, kdr, flk-1, or flt-1, to provide active immunity against angiogenesis. Therefore, this invention provides the first example of such anti-idiotypes being used to prevent or inhibit angiogenesis.

Blood vessels are formed by vasculogenesis, a process during which a primary capillary plexus is formed that is remodelled either by fusion or regression, and angiogenesis (also called neovascularization), a process in which vasculature is formed by new vessels sprouting from preexisting vessels and invading the developing organ. (Breier et al. 1996). Angiogenesis is an important process in the menstrual cycle in the endometrium, in pregnancy, and during neonatal growth. Angiogenesis is also important in wound healing and in the pathogenesis of a large variety of clinical diseases including tissue inflammation, arthritis, tumor growth, diabetic retinopathy, and macular degeneration by neovascularization of the retina. These clinical manifestations associated with angiogenesis are referred to as angiogenic diseases. (Folkman et al., Science, 235:442-447 (1987). Angiogenesis is generally absent in healthy adult or mature tissues, although it does occur in wound healing and in the corpus luteum growth cycle. See, for example, Moses et al., Science, 248:1408-1410 (1990).

Angiogenesis is required for tumor proliferation, since tumors need an adequate blood perfusion to obtain nutrients. Inhibiting angiogenesis by limiting vessel growth or selectively destroying proliferating endothelium would restrict tumor growth. Proposed methods of inhibiting angiogenesis include: (1) inhibition of release of "angiogenic molecules" such as basic-FGF (basic fibroblast growth factor), (2) neutralization of angiogenic molecules, such as by use of anti-basic-FGF antibodies, and (3) inhibition of endothelial cell response to angiogenic stimuli. Folkman et al., Cancer Biology, 3:89-96 (1992), have described several endothelial cell response inhibitors, including collagenase inhibitor, angiostatic steroids, fungal-derived angiogenesis inhibitors, platelet factor 4, thrombospondin, arthritis drugs such as D-penicillamine and gold thiomalate, vitamin D_3 analogs, alpha-interferon, and others that might be used to inhibit angiogenesis. For additional proposed inhibitors of angiogenesis, see Blood et al., Bioch. Biophys. Acta., 1032:89-8 (1990), Moses et al., Science 248:1408-1410 (1990), Ingber et al., Lab. Invest., 59:44-51 (1988), and U.S. Patent Nos. 5,092,885; 5,112,946; 5,192,744; and 5,202,352. Other new inhibitors of angiogenesis include angiostatin (O'Reilly et al., Cell 79:185-188 (Oct. 1994)) and endostatin.

The vascular endothelium is usually quiescent and its activation is tightly regulated during angiogenesis. Several factors have been implicated as possible regulators of angiogenesis *in vivo*. These include transforming growth factor (TGFb), acidic and basic fibroblast growth factor (aFGF and bFGF), platelet derived growth factor (PDGF), certain integrins, and vascular endothelial growth factor (VEGF) (Klagsbrun, M. and D'Amore. P. (1991) Annual Rev. Physiol. 53: 217-239).

VEGF, an endothelial cell-specific mitogen, acts as an angiogenesis inducer by specifically promoting the proliferation of endothelial cells. VEGF is a homodimeric glycoprotein consisting of two 23 kD subunits with structural similarity to PDGF. Four different monomeric isoforms of VEGF exist resulting from alternative splicing of mRNA. These include two membrane bound forms (VEGF206 and VEGF189) and two soluble forms (VEGF165 and VEGF121).

In all human tissues except placenta, VEGF165 is the most abundant isoform.

VEGF is expressed in embryonic tissues (Breier et al., Development (Camb.) 114:521 (1992)), macrophages, proliferating epidermal keratinocytes during wound healing (Brown et al., J. Exp. Med., 176:1375 (1992)), and may be responsible for tissue edema associated with inflammation (Ferrara et al., Endocr. Rev. 13:18 (1992)). *In situ* hybridization studies have demonstrated high VEGF expression in a number of human tumor lines including glioblastoma multiforme, hemangioblastoma, central nervous system neoplasms and AIDS-associated Kaposi's sarcoma (Plate, K. et al. (1992) Nature 359: 845-848; Plate, K. et al. (1993) Cancer Res. 53: 5822-5827; Berkman, R. et al. (1993) J. Clin. Invest. 91: 153-159; Nakamura, S. et al. (1992) AIDS Weekly, 13 (1)). High levels of VEGF were also observed in hypoxia induced angiogenesis (Shweiki, D. et al. (1992) Nature 359: 843-845).

The biological response of VEGF is mediated through its high affinity VEGF receptors which are selectively expressed on endothelial cells during embryogenesis (Millauer, B., et al. (1993) Cell 72: 835-846) and during tumor formation. VEGF receptors typically are class III receptor-type tyrosine kinases characterized by having several, typically 5 or 7, immunoglobulin-like loops in their amino-terminal extracellular receptor ligand-binding domains (Kaipainen et al., J. Exp. Med. **178**:2077-2088 (1993)). The other two regions include a transmembrane region and a carboxy-terminal intracellular catalytic domain interrupted by an insertion of hydrophilic interkinase sequences of variable lengths, called the kinase insert domain (Terman et al., Oncogene **6**:1677-1683 (1991). VEGF receptors include *flt-1*, sequenced by Shibuya M. et al., Oncogene 5, 519-524 (1990); *KDR*, described in PCT/US92/01300, filed February 20, 1992, and in Terman et al., Oncogene **6**:1677-1683 (1991); and *flk-1*, sequenced by Matthews W. et al. Proc. Natl. Acad. Sci. USA, **88**:9026-9030 (1991). *KDR* is the human form of *flk-1*.

High levels of flk-1 are expressed by endothelial cells that infiltrate gliomas (Plate, K. et al., (1992) Nature 359: 845-848). Flk-1 levels are specifically

upregulated by VEGF produced by human glioblastomas (Plate, K. et al. (1993) Cancer Res. 53: 5822-5827). The finding of high levels of flk-1 expression in glioblastoma associated endothelial cells (GAEC) indicates that receptor activity is probably induced during tumor formation since flk-1 transcripts are barely detectable in normal brain endothelial cells. This upregulation is confined to the vascular endothelial cells in close proximity to the tumor. Blocking VEGF activity with neutralizing anti-VEGF monoclonal antibodies (mAbs) resulted in an inhibition of the growth of human tumor xenografts in nude mice (Kim, K. et al. (1993) Nature 362: 841-844), indicating a direct role for VEGF in tumor-related angiogenesis.

Integrins are a class of cellular receptors known to bind extracellular matrix proteins, and therefore mediate cell-cell and cell-extracellular matrix interactions, called cell adhesion events. The integrin receptors constitute a family of proteins with shared structural characteristics of non-covalent heterodimeric glycoprotein complexes formed of α and β subunits.

Angiogenesis in tissues has been shown to require integrin $\alpha_v\beta_3$, and inhibitors of $\alpha_v\beta_3$ have been shown to inhibit angiogenesis (PCT Int'l. Application No. PCT/US95/03035, filed March 9, 1995). However, the use of anti-idiotypic antibodies mimicking any integrins, or any other angiogenesis targets, to inhibit angiogenesis has not been previously demonstrated.

Although angiogenesis related receptors are upregulated in tumor infiltrated vascular endothelial cells, the expression of these receptors is low in normal cells that are not associated with angiogenesis. Therefore, such normal cells would not be affected by inducing an immune response to such receptors to inhibit angiogenesis, and therefore to inhibit tumor growth.

An object of this invention is to provide a method of inhibiting an unwanted angiogenic condition, such as tumor angiogenesis, rheumatoid arthritis, diabetic retinopathy and psoriasis, by inducing an immune response in the subject against an angiogenic molecule. Another object of this invention is to provide

immunogens that are capable of inducing an immune response in a subject against an angiogenic molecule.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting an unwanted angiogenic condition in a mammal comprising treating the mammal with an effective amount of an immunogen that causes an immune response against a molecule that induces angiogenesis in the mammal.

The present invention also provides an immunogen that mimics a mammalian angiogenic molecule wherein the immunogen is not native to the mammal.

The invention also provides a method of inhibiting an unwanted angiogenic condition in a mammal comprising treating the mammal with an effective amount of a vector containing DNA that expresses an immunogen that causes an immune response against a molecule that induces angiogenesis in the mammal.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method of inhibiting an unwanted angiogenic condition in a mammal. The method comprises treating the mammal with an effective amount of an immunogen that causes an immune response against a molecule that induces or regulates angiogenesis in the mammal. The unwanted angiogenic condition may be tumor growth, arthritis, macular degeneration, psoriasis, or any other pathological angiogenic condition. The animal is preferably a mammal, which may be a human or an animal typically used for experimentation, such as mice, rats or rabbits.

In addition to providing the methods of the invention, the present invention also provides the immunogens used in these methods. The immunogens of

the invention unexpectedly induce an effective immune response when properly presented to the immune system. The immune response preferably inhibits, i.e. prevents, slows or stops, angiogenesis, and therefore inhibits or eliminates the pathological condition associated with angiogenesis, such as growth of tumors.

The immunogens of the invention may be any angiogenic molecule associated with the process of angiogenesis, such as, but not limited to vascular endothelial growth factor (VEGF), angiopoietin-1, angiopoietin-2, and basic fibroblast growth factor (bFGF). Further, the immunogen may be receptors associated with the process of angiogenesis, for example, flk-1, flt-1, and KDR; or integrins such as the vitronectin receptor $\alpha_v\beta_3$; or vascular endothelial cadherins (VE-Cadherin-1 and VE-Cadherin-2); TIE-1, TIE-2/Tek. However, these are examples of angiogenic molecules and receptors, and any angiogenic molecule or molecular target involved in angiogenesis may be utilized in the invention. The immunogen may be obtained from any animal or may be synthetically derived, providing it is substantially the same as that produced by the animal. The immunogen may also be introduced to any animal as naked DNA, or as a nucleic acid formulation, or as a plasmid containing DNA, which provide for the expression of a full length protein or a fragment thereof. (See U.S. Patent Nos. 5,589,466 and 5,630,796.) The immunogen may be a fragment of an antigen, epitope or antigenic determinant.

The immunogen used in the invention may be a small molecule not native to the mammal or a nucleic acid molecule not native to the mammal. Such small molecules or nucleic acids may be synthesized or isolated from an organism other than the mammal. The immunogen of the invention may also be a peptide molecule, or peptidomimetic, capable of illiciting an immune response against a molecules involved in angiogenesis. Such peptide molecules and peptidomimetics may be synthesized or isolated from an organism other than the mammal. Methods for screening small molecules, nucleic acid molecules, peptide molecules and peptidomimetics are well-known in the art. (See, for example, J. Biomolecular Screening, 1 (1), pg 27-31, 1996.)

The immunogen used in the method of the invention may be an antigen that is native to the mammal, and is modified to improve immunogenicity. The term "native" is defined herein as meaning autologous or homologous to an animal. In other words, the native antigens of the invention are "self" proteins and therefore typically non-immunogenic in the animal from which they are derived. The antigen may be purified or substantially purified. The immunogens may also be not native, meaning foreign, to a mammal of the invention, such as small molecules or nucleic acid molecules that are not native to the mammal.

The immunogens of the invention are modified in various ways known in the art, such as by conjugating or genetically fusing the immunogen to an immunogenic reagent. Conjugation or fusion of the immunogen to an immunogenic reagent can stimulate an immune response to the immunogen. The conjugates and fused molecules of this invention can be prepared by any of the known methods for coupling or fusing antigens to carriers or fusion molecules. The conjugates may also be prepared recombinantly as fusion proteins by methods well-known in the art. The preferred method of conjugation is covalent coupling whereby the antigen is bound directly to the immunogenic reagent. Preferred immunogenic reagents include polysaccharides (U.S. Patent No. 5,623,057), and peptidoglycans (U.S. Patent No. 5,153,173). These U.S. Patents, as well as all such patents presented in the instant specification, are herein incorporated by reference.

Another method of modifying the immunogens of the invention is for them to be bound or genetically fused with a cytokine, lymphokine, hormone or growth factor (U.S. Patent No. 5,334,379). Examples of such molecules include, but are not limited to, interferons, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6 and IL-7 (U.S. Patent No. 5,334,379). As stated above, these U.S. Patents are herein incorporated by reference.

Another method of modifying the immunogens of the invention is haptenization (chemically linking) of the antigen. A hapten is a substance having the ability to, when coupled with a protein, elicit an immune response. The immunogen of

the invention can itself be haptenized , or can be bound to hapten-modified proteins. (U.S. Patent Nos. 4,778,752 and 5,290,551).

An additional method of modifying the immunogens of the invention is glycosylation of the antigens or glycosylation of the carrier molecules of the antigens (see U.S. Patent Nos. 5,484,735 and 4,629,692).

Furthermore, peptidomimetic compounds, i.e., compounds which mimic the activity of peptides, may be used in modification of the immunogens of the invention (U.S. Patent Nos. 5,386,011 and 5,153,173). Additionally, peptidomimetics, which are immunogenic and illicit an immune response against molecules involved in angiogenesis are themselves useful as immunogens in the invention.

Modification of the immunogens of the invention by bonding the immunogen with a Major Histocompatibility Complex (MHC) antigen, forming a complex that is also useful in this invention. (U.S. Patent No. 4,478,823). The source of such MHC antigens and the methods of bonding the immunogens of the invention to the MHC antigens are detailed in the cited U.S. Patent No. 4,478,823, which is herein incorporated by reference.

PREPARATION OF IMMUNOGENS

Many of the angiogenic protein molecules of the invention are known, and may be obtained in natural or recombinant form by known methods. Newly identified, as well as molecules not yet identified may also be obtained and used as part of the invention. Methods of obtaining such molecules include isolating the angiogenic protein directly from cells; isolating or synthesizing DNA encoding the protein and using the DNA to produce recombinant protein; and synthesizing the protein chemically from individual amino acids.

The entire angiogenic molecule gene or fragments of the gene may, for example, be isolated by using the known DNA sequence to construct

oligonucleotide probes. To do so, DNA restriction fragments are identified by Southern hybridization using labelled oligonucleotide probes derived from the known sequence.

Alternatively, DNA encoding the angiogenic molecule may be synthesized from individual nucleotides. Known methods for synthesizing DNA include preparing overlapping double-stranded oligonucleotides, filling in the gaps, and ligating the ends together.

The DNA prepared as described above may be amplified by polymerase chain reaction (PCR). Alternatively, the DNA may be amplified by insertion into a cloning vector, which is transfected into a suitable host cell, from which the DNA may be recovered. See, generally, Sambrook et al, "Molecular Cloning," Second Edition, Cold Spring Harbor Laboratory Press (1987).

Recombinant methods well known in the art may be used for preparing the protein. Briefly, the angiogenic molecule-encoding DNA is inserted into an expression vector, which is transfected into a suitable host. The DNA is expressed, and the protein is harvested. See Sambrook et al., Id.

Equivalents of the angiogenic protein may also be used in the invention. Such equivalents include analogs that induce an immune response comparable to that of the protein. In addition, such equivalents are immunologically cross-reactive with their corresponding protein. The equivalent may, for example, be a fragment of the protein, or a substitution, addition or deletion mutant of the protein.

The angiogenic protein fragment preferably contains sufficient amino acid residues to define an epitope of the antigen. The fragment may, for example, be a minigene encoding only the epitope. Methods for isolating and identifying immunogenic fragments from known immunogenic proteins are described by Salfeld et al. in J. Virol. 63, 798-808 (1989) and by Isola et al. in J. Virol. 63, 2325-2334 (1989).

If the fragment defines a suitable epitope, but is too short to be immunogenic, it may be conjugated to a carrier molecule. Some suitable carrier molecules include keyhole limpet hemocyanin, Ig sequences, TrpE, and human or bovine serum albumen. Conjugation may be carried out by methods known in the art. One such method is to combine a cysteine residue of the fragment with a cysteine residue on the carrier molecule.

Equivalent proteins have equivalent amino acid sequences. An amino acid sequence that is substantially the same as another sequence, but that differs from the other sequence by one or more substitutions, additions and/or deletions, is considered to be an equivalent sequence. Preferably, less than 25%, more preferably less than 10%, and most preferably less than 5% of the number of amino acid residues in a sequence are substituted for, added to, or deleted from the proteins of the invention.

For example, it is known to substitute amino acids in a sequence with equivalent amino acids. Groups of amino acids generally considered to be equivalent are:

(a) Ala(A) Ser(S) Thr(T) Pro(P) Gly(G);
(b) Asn(N) Asp(D) Glu(E) Gln(Q);
(c) His(H) Arg(R) Lys(K);
(d) Met(M) Leu(L) Ile(I) Val(V); and
(e) Phe(F) Tyr(Y) Trp(W).

The present invention also provides anti-idiotypic antibodies or fragments thereof that mimic angiogenic molecules. An "antibody" in accordance with the present specification is defined broadly as a protein that specifically binds to an epitope. The antibodies may be polyclonal, or, preferably, monoclonal. The angiogenic molecule may be any molecule involved in the process of angiogenesis, such as, but not limited to, vitronectin, vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF), flt-1, flk-1, KDR, angiopoietin 1 and 2, VE-Cadherin 1 and 2, TIE-1 and TIE-2/TEK.

The modified immunogens and anti-idiotypic antibodies of the invention elicit an immune response in an animal against angiogenesis. The animal is preferably a mammal, such as a rabbit, rat, or mouse. Preferably, the animal is a human. An immune response means production of antibodies, i.e. humoral, and/or a cell-mediated response, such as a T-cell response including helper and cytotoxic T cell responses.

The anti-idiotypic antibody of the invention is directed against any antibody, which is itself directed against antigenic determinants of receptors to angiogenic molecules, or the angiogenic molecule itself. Such antibodies are known in the art, such as the anti-flk-1 antibody, DC101, described in PCT/US95/01678, filed February 10, 1995; or anti-$\alpha_v\beta_3$ antibody, i.e. LM609 monoclonal antibody described in PCT Int'l. Application No. PCT/US95/03035, filed March 9, 1995. Such angiogenic molecules, such as VEGF, are also known. Further, anti-receptor and anti-angiogenic molecule antibodies may be obtained by methods known in the art such as those described below.

PREPARATION OF ANTIBODIES

The polyclonal and monoclonal antibodies of the invention may be produced by methods known in the art. These methods include the immunological method described by Kohler and Milstein in Nature 256, 495-497 (1975) and Campbell in "Monoclonal Antibody Technology, The Production and Characterization of Rodent and Human Hybridomas" in Burdon et al., Eds., Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier Science Publishers, Amsterdam (1985); as well as by the recombinant DNA method described by Huse et al in Science 246, 1275-1281 (1989).

The antibody may be prepared in any mammal, including mice, rats, rabbits, goats and humans. The antibody may be a member of one of the following immunoglobulin classes: IgG, IgM, IgA, IgD, or IgE, and the subclasses thereof.

In one embodiment of the invention, a panel of syngeneic anti-idiotypic

monoclonal antibodies is developed using DC101 mAb and anti-$\alpha_v\beta_3$ mAb, which are both monoclonal antibodies to angiogenic receptors. An anti-idiotypic monoclonal antibody bearing the internal image of the determinants recognized by the DC101 monoclonal antibody and anti-$\alpha_v\beta_3$ monoclonal antibody is identified by determining which anti-idiotypic monoclonal antibody can induce anti-$\alpha_v\beta_3$ mAb and anti-Flk-1 antibodies in the test animal.

Further, one embodiment of the invention provides a cell which produces the anti-idiotypic antibody of the invention. This cell may be any cell, including genetically engineered bacterial cells such as *E. coli* cells containing DNA to produce the antibody as well as the more typical mammalian cells such as B cells hybridized with murine myeloma cell lines using standard fusion procedures (Kearney, J. F. et al., 1981, Eur. J. Immunol. 11:877). Methods for producing hybridomas, which have the capacity to produce a monoclonal antibody, are well known in the art. (see Niman et al. Proc. Natl. Acad. Sci. USA 80:4949-4953 (1983) and Galfre et al., Meth. Enzymol., 73:3-46 (1981)).

Functional Equivalents of Antibodies

The invention also includes functional equivalents of the antibodies described in this specification. Functional equivalents have binding characteristics comparable to those of the antibodies, and include, for example, chimerized, humanized and single chain antibodies as well as fragments thereof. Diabodies may also be functional equivalents of the antibodies of this invention. Methods of producing such functional equivalents are disclosed in PCT Application No. WO 93/21319, European Patent Application No. EPO 239,400; PCT Application Wo 89/09622; European Patent Application No. 338,745; and European Patent Application EPO 332,424.

Functional equivalents include polypeptides with amino acid sequences substantially the same as the amino acid sequence of the variable or hypervariable regions of the antibodies of the invention. "Substantially the

same" amino acid sequence is defined herein as a sequence with at least 70% percent homology to an amino acid sequence of an antibody of the invention, as determined by the FASTA search method in accordance with Pearson and Lipman, Proc. Natl. Acad. Sci. USA 85, 2444-2448 (1988).

Chimerized antibodies preferably have constant regions derived substantially or exclusively from human antibody constant regions and variable regions derived substantially or exclusively from the sequence of the variable region from a mammal other than a human.

Humanized antibodies preferably have constant regions and variable regions other than the complementarity determining regions (CDRs) derived substantially or exclusively from the corresponding human antibody regions and CDRs derived substantially or exclusively from a mammal other than a human.

Suitable mammals other than a human include any mammal from which monoclonal antibodies may be made, such as a rabbit, rat, mouse, horse, goat, or primate.

Single chain antibodies or Fv fragments are polypeptides which consist of the V region of the heavy chain of the antibody linked to the V region of the light chain with or without an interconnecting linker. This comprises the entire antibody combining site, and is the minimal antibody binding site. These chains may be produced in bacteria.

Functional equivalents further include fragments of antibodies that have the same or binding characteristics comparable to those of the whole antibody. Such fragments may contain one or both Fab fragments or the $F(ab')_2$ fragment. Preferably the antibody fragments contain all six complementarity determining regions of the whole antibody, although fragments containing fewer than all of such regions, such as three, four or five CDRs, may also be functional.

Diabodies are examples of additional functional equivalents. A diabody is an antibody fragment which has two antigen binding sites and can be a bivalent or bispecific fragment. Bispecific diabodies are heterodimers of two 'crossover' scFv fragments in which the variable light and variable heavy domains of the two antibodies are present on different polypeptide chains. (Carter and Merchant, Current Opinions in Biotechnology (8):449-454, 1997.)

Further, the functional equivalents may be or may combine members of any one of the following immunoglobulin classes: IgG, IgM, IgA, IgD, or IgE, and the subclasses thereof.

Gene Therapy and Vector Delivery

Intracellularly expressed antibodies, referred to as "intrabodies" can be designed to bind and inactivate target molecules inside cells. The genes encoding can be expressed intracellularly. The specific and high-affinity binding properties of antibodies, combined with their ability to be stably expressed in precise intracellular locations inside mammalian cells, provides molecules for gene therapy applications. (Marasco, W., Gene Ther (4) 1, p11-5, 1997).

Genes encoding immunogens not native to the mammal may be introduced into mammalian cells, particularly endothelial cells, by methods known in the art. Such methods have been described, for example, in Mulligan, et al., U.S. patent 5,674,722. The methods described in Mulligan, et al., U.S. patent 5,674,722 for preparing vectors useful for introducing genes into mammalian cells, particularly endothelial cells, are incorporated herein by reference.

The immunogen may be presented to the immune system by a vehicle. For example, the immunogen may be present on the surface of an antigen presenting cell, such as a dendritic cell, or combined with a pharmaceutically acceptable carrier or adjuvant.

Antigen presenting cells are generally eukaryotic cells with major histocompatibility complex (MHC), either Class I or Class II, gene products at their cell surface. For the purposes of this specification, antigen presenting cells also include recombinant eucaryotic cells, such as peripheral blood cells, and recombinant bacterial cells. Some examples of antigen presenting cells as defined by this specification include dendritic cells, macrophages that are preferably MHC Class II positive, monocytes that are preferably MHC Class II positive, and lymphocytes. (Also see U.S. Patent No. 5,597,563).

In one embodiment of the subject invention, the antigen presenting cell is a recombinant eucaryotic cell that expresses exogenous DNA encoding the antigen of the invention. The recombinant eucaryotic cell may be prepared *in vivo* or *in vitro*.

Suitable cloning/expression vectors for inserting DNA into eucaryotic cells include well-known derivatives of SV-40, adenovirus, cytomegalovirus (CMV), and retrovirus-derived DNA sequences. Any such vectors, when coupled with vectors derived from a combination of plasmids and phage DNA, i.e. shuttle vectors, allow for the cloning and/or expression of protein coding sequences in both procaryotic and eucaryotic cells.

Other eucaryotic expression vectors are known in the art, e.g., P.J. Southern and P. Berg, J. Mol. Appl. Genet. 1, 327-341 (1982); S. Subramani et al, Mol. Cell. Biol. 1, 854-864 (1981); R.J. Kaufmann and P.A. Sharp, "Amplification And Expression Of Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene," J. Mol. Biol. 159, 601-621 (1982); R.J. Kaufmann and P.A. Sharp, Mol. Cell. Biol. 159, 601-664 (1982); S.I. Scahill et al, "Expression and Characterization of the Product of a Human Immune Interferon DNA Gene in Chinese Hamster Ovary Cells," Proc. Natl. Acad. Sci. USA 80, 4654-4659 (1983); G. Urlaub and L.A. Chasin, Proc. Natl. Acad. Sci. USA 77, 4216-4220, (1980).

The immunogens of the invention may also be presented to the immune

system on the surface of recombinant bacterial cells. A suitable recombinant bacterial cell is an avirulent strain of Mycobacterium bovis, such as bacille Calmette-Guerin (BCG), or an avirulent strain of Salmonella, such as S. typhimurium. The recombinant bacterial cells may be prepared by cloning DNA comprising the active portion of the antigen in an avirulent strain, as is known in the art; see, for example, Curtiss et al., Vaccine 6, 155-160 (1988) and Galan et al., Gene 94, 29-35 (1990) for preparing recombinant Salmonella and Stover, C.K. et al., Vaccines 91, Cold Spring Harbor Laboratory Press, pp. 393-398 (1991) for preparing recombinant BCG.

Cloning vectors may comprise segments of chromosomal, non-chromosomal and synthetic DNA sequences. Some suitable prokaryotic cloning vectors include plasmids from E. coli, such as colE1, pCR1, pBR322, pMB9, pUC, pKSM, and RP4. Prokaryotic vectors also include derivatives of phage DNA such as M13, fd, and other filamentous single-stranded DNA phages.

Vectors for expressing proteins in bacteria, especially E. coli, are also known. Such vectors include the pK233 (or any of the tac family of plasmids), T7, and lambda P_L. Examples of vectors that express fusion proteins are PATH vectors described by Dieckmann and Tzagoloff in J. Biol. Chem. 260, 1513-1520 (1985). These vectors contain DNA sequences that encode anthranilate synthetase (TrpE) followed by a polylinker at the carboxy terminus. Other expression vector systems are based on beta-galactosidase (pEX); lambda P_L; maltose binding protein (pMAL); glutathione S-transferase (pGST) - see Gene 67, 31 (1988) and Peptide Research 3, 167 (1990).

The expression vectors useful in the present invention contain at least one expression control sequence that is operatively linked to the DNA sequence or fragment to be expressed. The control sequence is inserted in the vector in order to control and to regulate the expression of the cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of fd coat protein, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late

promoters of SV40, and other sequences known to control the expression of genes in prokaryotic or eukaryotic cells and their viruses or combinations thereof.

The immunogens of the invention may also be combined with a suitable medium. Suitable media include pharmaceutically acceptable carriers, such as phosphate buffered saline solution, liposomes and emulsions.

The immunogens may also be combined with pharmaceutically acceptable adjuvants that may enhance the immune response, such as muramyl peptides, lymphokines, such as interferon, interleukin-1 and interleukin-6, or bacterial adjuvants. The adjuvant may comprise suitable particles onto which the immunogen is adsorbed, such as aluminum oxide particles. These compositions containing adjuvants may be prepared as is known in the art.

An example of a bacterial adjuvant is BCG. When used as an antigen presenting cell as described above, recombinant BCG may additionally act as its own adjuvant. In this case, additional adjuvant may not be needed although one or more additional adjuvants may optionally be present. When used in its natural (non-recombinant) state, BCG acts solely as an adjuvant by being combined with the immunogen or anti-idiotypic antibody, resulting in a form that induces an effective immune response.

The immunogen or immunogen composition may be administered to a mammal by methods known in the art. Such methods include, for example, intravenous, intraperitoneal, subcutaneous, intramuscular, topical, or intradermal administration.

Assays for Determining the Level of Antibody to Anti-Receptor Antibody or Receptor in Cells

The level of anti-receptor antibody or to receptor in a sample may be determined by assays known in the art using the anti-idiotypic antibody of the

invention. The results of these assays may be used for diagnostic purposes. The target antibody or antigen may be immobilized on a support either indirectly by using an anti-target antibody or directly to the support. Since the anti-idiotypic antibody mimics the receptor and will compete with and thereby inhibit the ability of the receptor to bind with anti-receptor antibodies, a competitive assay may be used to measure the concentration of receptor or anti-receptor antibodies by correlating the level of anti-idiotypic antibody binding to the concentration of receptor or anti-receptor antibodies.

A variety of assays are available for detecting proteins with labeled antibodies. In a one-step assay, the target molecule, if it is present, is immobilized and incubated with a labeled anti-idiotypic antibody. The labeled anti-idiotypic antibody binds to the immobilized target molecule. After washing to remove unbound molecules, the sample is assayed for the presence of the label.

In a two-step assay, immobilized target molecule is incubated with an unlabeled anti-idiotypic antibody. The target molecule-unlabeled anti-idiotypic antibody complex, if present, is then bound to a second, labeled antibody that is specific for the unlabeled antibody. The sample is washed and assayed for the presence of the label, as described above.

The choice of marker used to label the antibodies will vary depending upon the application. However, the choice of marker is readily determinable to one skilled in the art. The labeled antibodies may be polyclonal or monoclonal. In a preferred embodiment, the antibody is monoclonal.

Purification Methods

The invention also provides a method of purifying anti-receptor antibodies in a sample comprising contacting the sample with an anti-idiotypic antibody of the invention, isolating the complex between anti-receptor antibodies from the sample and the anti-idiotypic antibody, and recovering the anti-receptor

antibodies from the complex. The anti-idiotypic antibody is preferably immobilized.

Experimental Examples

Dendritic cells (DC): DC were isolated using the following protocol. Bone marrow from C57BL6 mice was depleted of red blood cells by treatment in 0.5% amonium chloride, followed by treatment with a combination of monoclonal antibodies specific for CD4, CD8, Ia, granulocytes and then with rabbit complement. The remaining cells were cultured in RPMI 1640 containing 10% FBS as well as GM-CSF and IL-4. Three days later, nonadherent cells were discarded and adherent cells cultured for 4 more days. Adherent cells were then transferred to a new plate and cultured for another 3 days before harvesting for use as antigen presenting cells.

Pulsing of DC with Flk-1AP antigen: DC were washed twice in serum-free AIMV media and incubated overnight in AIMV with 100ug/ml of an affinity-purified fusion protein of Flk-1 and alkaline phosphatase (Flk-1AP). The cells were then washed twice in AIMV before being used for vaccination.

Vaccination of mice with DC pulsed with Flk-1AP: Lewis lung carcinoma metastasis model was used to assess the antitumor effect of vaccination with DC pulsed with Flk-1AP. Briefly, C57BL6 mice were injected either intravenously or intraperiteneally, with each mouse receiving 1 x 10^5 Flk-1 AP-pulsed DC at Day 0. Seven days later, each mouse was inoculated intra-footpad with 1x 10^5 D122 cells. Visible tumors were surgically removed at Day 10. During this period, mice received two boost vaccinations every ten days. At Day 60, mice were sacrificed and lungs removed for weighing and assessment of metastases. As a control, a group of mice were vaccinated with DC alone.

Experimental Results

Inhibition of tumor metastases: Mice vaccinated with DC pulsed with Flk-

1AP developed a significantly smaller number of tumors in the lungs compared to the groups vaccinated with DC alone. Comparably, the average weight of the lungs from the Flk-AP group was significantly lower than the controls. In both instances, the number of tumors present and the weight of the lungs of mice vaccinated with DC pulsed with Flk-1AP was less than half of the control group.

We Claim:

1. A method of inhibiting an unwanted angiogenic condition in a mammal in need thereof comprising treating the mammal with an effective amount of an immunogen that causes an immune response against a molecule that induces angiogenesis in the mammal.

2. The method of claim 1 wherein the unwanted angiogenic condition is tumor growth.

3. The method of claim 1 wherein the unwanted angiogenic condition is arthritis.

4. The method of claim 1 wherein the unwanted angiogenic condition is macular degeneration.

5. The method of claim 1 wherein the unwanted angiogenic condition is psoriasis.

6. The method of claim 1 wherein the mammal is a human.

7. The method of claim 1 wherein the immunogen is an antigen that is native to the mammal, and that is modified to improve immunogenicity.

8. The method of claim 7 wherein the antigen is a haptenized antigen.

9. The method of claim 7 wherein the antigen is conjugated to an immunogenic compound.

10. The method of claim 7 wherein the antigen is combined with an adjuvant.

11. The method of claim 1 wherein the immunogen is bound to a MHC

Class I Restricted Antigen forming a complex not native to the mammal.

12. The method of claim 1 wherein the immunogen is bound to a MHC Class II
Restricted Antigen forming a complex not native to the mammal.

13. The method of claim 7 wherein the antigen is substantially purified.

14. The method of claim 1 wherein the immunogen is an anti-idiotypic monoclonal antibody.

15. The method of claim 1 wherein the immunogen is a synthetic peptide not native to the mammal.

16. The method of claim 1 wherein the immunogen is a small molecule not native to the mammal.

17. The method of claim 1 wherein the immunogen is expressed on an antigen-presenting cell not native to the mammal.

18. The method of claim 17 wherein the antigen-presenting cell is a dendritic cell.

19. The method of claim 1 wherein the immunogen is a nucleic acid molecule not native to the mammal.

20. The method of claim 1 wherein the a molecule that induces angiogenesis is FLK-1.

21. The method of claim 1 wherein the a molecule that induces angiogenesis is KDR.

22. The method of claim 1 wherein the a molecule that induces

angiogenesis is FLT-1.

23. The method of claim 1 wherein the a molecule that induces angiogenesis is VEGF.

24. The method of claim 1 wherein the a molecule that induces angiogenesis is a Vascular Endothelial Cadherin.

25. The method of claim 1 wherein the a molecule that induces angiogenesis is TIE-1.

26. The method of claim 1 wherein the a molecule that induces angiogenesis is TIE-2/Tek.

27. The method of claim 1 wherein the a molecule that induces angiogenesis is an integrin.

28. The method of claim 27 wherein the integrin is alphaVbeta3.

29. The method of claim 1 wherein the a molecule that induces angiogenesis is bFGF.

30. The method of claim 1 wherein the a molecule that induces angiogenesis is vitronectin.

31. An immunogen that mimics a mammalian angiogenic molecule wherein the immunogen not native to the mammal.

32. The immunogen of claim 31, wherein the immunogen is an anti-idiotypic antibody.

33. The immunogen of claim 31, wherein the immunogen is an isolated peptide.

34. The immunogen of claim 31, wherein the immunogen is an isolated small molecule.

35. The immunogen of claim 31, wherein the immunogen is a nucleic acid molecule.

36. The immunogen of claim 31, wherein the immunogen is an antigen-presenting cell.

37. The immunogen of claim 36, wherein the antigen-presenting cell is a dendritic cell.

38. The immunogen of claim 31, wherein the immunogen is a MHC Class I Restricted Antigen complex.

39. The immunogen of claim 31, wherein the immunogen is a MHC Class II Restricted Antigen complex.

40. The immunogen of claim 32, wherein the antibody is monoclonal.

41. The immunogen of claim 40, comprising a fragment of the monoclonal antibody.

42. A cell which produces the anti-idiotypic antibody of claim 40.

43. A cell of claim 42 that is a hybridoma.

44. A polypeptide comprising an amino acid sequence that is substantially the same as, the amino acid sequence of the variable region of the monoclonal antibody of claim 40, and that has the same binding specificity.

45. A nucleic acid that encodes the hypervariable region of the antibody of

claim 40.

46. A nucleic acid that encodes the polypeptide of claim 44.

47. A nucleic acid that hybridizes under stringent conditions to the nucleic acid of claim 45 and that encodes a polypeptide having the same binding specificity as the hypervariable region encoded by the nucleic acid of claim 45.

48. A chimeric antibody comprising the polypeptide of claim 44.

49. A chimeric antibody of claim 48 comprising an amino acid sequence of a human antibody constant region and an amino acid sequence of a non-human antibody variable region.

50. A chimeric antibody of claim 49, wherein the non-human variable region is murine.

51. A polypeptide which comprises an amino acid sequence which is substantially the same as the amino acid sequence of the hypervariable region of the monoclonal antibody of claim 40 and that has the same binding specificity.

52. A nucleic acid that encodes the polypeptide of claim 51.

53. A humanized antibody or a fragment thereof comprising the polypeptide of claim 51.

54. The humanized antibody of claim 53 comprising amino acid sequences of framework and constant regions from a human antibody, and an amino acid sequence of a non-human antibody hypervariable region.

55. The humanized antibody of claim 54, wherein the amino acid sequence

of the hypervariable region is murine.

56. A method of inhibiting an unwanted angiogenic condition in a mammal in need thereof comprising treating the mammal with an effective amount of a vector that expresses an immunogen that causes an immune response against a molecule that induces angiogenesis in the mammal.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05164

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/02; C12N 15/00, 9/96; A01N 63/00; A61K 39/395, 39/00.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 435/325, 320.1, 188; 424/93.1, 130.1, 131.1, 134.1, 139.1, 141.1, 192.1.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSIS, CAPLUS, MEDLINE, SCISEARCH.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TAKAHASHI et al. Inhibition of cell growth and tumorigenesis of human glioblastoma cells by a neutralizing antibody against human basic fibroblast growth factor. FEBS. August 1991, Vol. 288, No. 1-2, pages 65-71, especially abstract.	1-6, 16, 29
X	KIM et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. Nature. 29 April 1993, Vol. 362, No. 6423, pages 841-844, especially abstract.	1-6, 16, 23
X	DAVIS et al. Identification of a role of the vitronectin receptor and protein kinase C in the induction of endothelial cell vascular formation. Journal of Cellular Biochemistry. 1993, Vol. 51, pages 206-218, especially abstract.	1-6, 16, 30

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
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International application No.
PCT/US99/05164

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OLIVER et al. Suppression of collagen-induced arthritis by an angiogenesis inhibitor, AGM-1470, in combination with cyclosporin: reduction of vascular endothelial growth factor (VEGF). Cellular Immunology. 1995, Vol. 166, pages 196-206, especially page 196.	1-6, 15, 16, 23
Y	YAMADA et al. Monoclonal antibody and synthetic peptide inhibitors of human tumor cell migration. Cancer Research. 01 August 1990, Vol. 50, No. 15, pages 4485-4496, especially page 4485.	1-6, 15, 16, 27, 28
Y	BROOKS et al. Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. Science. 22 April 1994, Vol. 264, No. 5158, pages 569-571, whole document.	1-6, 16, 27, 28
X, P --- Y, P	US 5,863,538 A (THORPE et al) 26 January 1999, whole document.	1, 2, 7 --- 3-6, 8-13, 17-19, 31, 33, 34
Y	US 5,334,379 A (PILLAI et al.) 02 August 1994, whole document.	9, 10
Y	US 5,589,466 A (FELGNER et al) 31 December 1996, whole document.	19, 31, 34, 35, 56
Y	US 4,778,752 A (CURTISS et al) 18 October 1988, whole document.	8
Y	US 4,478,823 A (SANDERSON) 23 October 1984, whole document.	11, 12, 38, 39
Y	US 5,580,563 A (TAM) 03 December 1996, whole document.	17, 18, 36, 37
Y	CHEN et al. Human high molecular weight melanoma-associated antigen mimicry by mouse antiidiotypic monoclonal antibody MK2-23. Journal of Immunology. 01 August 1991, Vol. 147, No. 3, pages 1082-1090, whole document.	14, 31, 32, 40-43

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.1; 435/325, 320.1, 188; 424/93.1, 130.1, 131.1, 134.1, 139.1, 141.1, 192.1.